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THE USE OF B. WELCHII IN THE PREPARATION OF SUGAR-FREE CULTURE MEDIUM

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The vigor of fermentation of B. welchii suggested that it might prove superior to the more commonly used aerobic organisms in the elimination of muscle sugar from mediums intended for the study of the fermentation reactions of bacteria.

That muscle sugar may confuse the interpretation of fermentation reactions has been recognized for some time. Smith,[1] in 1895, mentioned that the muscle sugar content might vary from a trace to 0.3% and concluded, in accord with physiologists, that it is mainly glucose since it is attacked by bacteria that do not ferment lactose or saccharose. Smith [2] emphasized the necessity of eliminating muscle sugar from meat infusion used in the preparation of differential carbohydrate medium, so that now it is a common laboratory procedure to use a so-called "sugar-free" medium as the basis for fermentation tests with known carbohydrates. But the details of preparation vary in different laboratories.

In some laboratories the fermenting culture is mixed with the ground meat when it is put to soak, and incubated at 37 C. overnight. In this method there is, of course, always a mixed bacterial flora. The mass is boiled to coagulate the albumins and kill the bacteria. Straining removes the former and filtration through paper most of the latter, but the product is nearly always somewhat turbid, and if clarity is desired, an absorbent, such as talc or diatomaceous earth, must be added previous to filtration. If the fermentation has proceeded too far, there is likely to be great difficulty in filtration, owing to the colloidal nature of the liquid. In such cases, acidification and reheating provide effective means for securing a filtrate, which, however, may still have to be clarified by the aid of an adsorbent as already mentioned.

In other laboratories it is customary to secure the infusion in the usual manner of cold soaking, boiling, and filtration, after which the

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[1] Centralbl. f. Bakteriol., 1895, 18, p. 1.

[2] Wilder Quarter Century Book, 1893, p. 187; Centralbl. f. Bakteriol., 1890, 7, p. 502; Jour. Exper. Med., 1897, 2, p. 543.

muscle juice is sterilized and then fermented out with a pure culture. The objection to this practice is that the medium at this stage is deficient in nutrients so that growth of the fermenting organism is never vigorous. A better plan is to ferment out the sugar after the peptone has been added, and this procedure has the added advantage of removing traces of sugar, which may be present in the peptone also.[3] This has been our own procedure in the experiments shortly to be described, except in two instances in which we used sterilized meat mashes to which peptone had been added, but from which the meat had not been strained.

Most bacteriologists, following the suggestion of Smith, have used Bact. coli as the fermenting culture. Bact. coli communior ferments a greater variety of carbohydrates than most other aerobic organisms. The strain used in our work was derived from human feces and fermented with acid and gas formation, glucose, lactose, saccharose and glycerol but not inulin or salicin. It has no liquefactive action on gelatin and gives a positive methyl red test and a negative Voges-Proskauer test.

If we assume that all of the muscle sugar consists of hexoses, versatility in the fermentation reactions is not a prerequisite in the culture selected.

Thus, Dr. K. F. Meyer of the Hooper Foundation for Medical Research, San Francisco, has used a culture designated Bact. saccharolyte for several years in the preparation of "sugar-free" medium. The only history of this culture that is available indicates that it came from Dr. Ernst of Harvard Medical School in 1911 and was studied by Dr. Rivas at the University of Pennsylvania. It is a coliform, gram-negative, nonsporulating rod which ferments with acid and gas production, glucose, saccharose, and salicin and gives a positive methyl red test. It does not ferment lactose, inulin or glycerol. It does not liquefy gelatin and gives a negative Voges-Proskauer test. It should be regarded as most closely related to the paratyphoid-enteritidis group of organisms, from which it differs in the fermentation of saccharose.

EXPERIMENTAL WORK

The experiments described in this paper deal with a comparison of B. welchii with Bact. coli and Bact. saccharolyte. The strain used was our B. welchii, No. 2, which was received from the American Museum of Natural History, May 2, 1916, under their No. B 521.

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[3] Colway: Science, 1915, 41, p. 662.

Dr. J. P. Simonds had recovered this culture about 1912 from stools as No. 16 in his series. When received by us it was free from aerobic contamination and gave a characteristic stormy fermentation of milk, but the presence of motile rods and the production of a black deposit in brain medium indicated contamination with a putrefactive anaerobe. This organism was isolated and identified as B. sporogenes. It bears No. 72 in our series. The Welch bacillus was also isolated free from contamination by four times repeated streaking and picking of well isolated colonies from the surface of blood-agar slants and twice repeated picking of isolated colonies from the depths of deep meat-infusion agar.[4] The culture forms central spores (sparingly) that do not swell the rod, the organisms are nonmotile, coagulated albumin is not liquefied, brain is not digested and only blackened in the presence of added iron ions, gelatin is liquefied and milk is fermented with vigorous production of gas and slight acid.

In studying the preparation of "sugar-free" broth, we have considered the possible applicability of several criteria of the presence of sugar, among which may be mentioned chemical tests for glucose, acid production as indicated by changes in titer and in P_H, and in one experiment, the formation of gas by refermentation.

Tests for carbohydrates based on the principle of reduction in an alkaline solution were tried. Fehling's test and the more recently devised Folin-McEllroy reagent[5] were used. It was found from the first, however, that culture broth contains some ingredient or ingredients which interfere with copper reduction with either of the above reagents even in the presence of as high as 1% glucose.

On the supposition that the protein of the medium had much to do with this interference, we undertook to eliminate this by various methods. Picric acid[6] was quite successful as a precipitant, but the resultant deep yellow color of the filtrate would not permit its use where observation of the color changes is essential.

Precipitation of the protein by the mercury reagent of Benedict and Ostenberg[7] was tried next. This reagent, when applied to urine, has the advantage not only of precipitating nitrogenous compounds as completely as possible, but also other possible interfering substances such as polyphenols, and glycuronic acid. The wide range of activity of this reagent in urine suggested its efficacy when applied to culture broth. A heavy precipitate and clear filtrate were obtained from broth treated with this reagent. Subsequent tests of this filtrate with the copper reagents failed to give positive results with either "normal" or "sugar-free" broth. After the addition of a minimum of 0.1% glucose to either filtrate, however, positive results were obtained. The conclusion was that the sugar content of ordinary broth was below the range of sensitivity of either of the reagents used and that, therefore, these qualitative sugar tests were not applicable to our purpose.

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[4] Hall: Jour. Infect. Dis., 1920, 27, p. 576.

[5] Jour. Biol. Chem., 1918, 33, p. 513.

[6] Lewis and Benedict: Jour. Biol. Chem., 1915, 20, p. 61.

[7] Ibid., 1918, 34, p. 195.

We have also made some preliminary tests of the method of blood sugar analysis outlined by Folin and Wu[8] in reference to the sugar content of broth, but further work will be necessary for the proper interpretation of the results.

Of the various criteria that may be used to guide the preparation of sugar-free broth the changes in reaction, measured either in terms of P_H or by titration, have seemed most reliable. The peculiar advantages of each appear in the charts. Our interpretation of changes in reaction toward acidity as indicative of fermentation, i. e., sugar splitting, and of changes in reaction toward alkalinity as indicative of proteolysis, is based on Kendall's conception[9] that "fermentation takes precedence over putrefaction." It is our belief that the peak of a curve plotted on a basis of acidity during the growth period indicates the "sugar-free" point, and when this point is reached the incubation should be stopped, for the culture has then reached the termination of its carbohydrate metabolism and the initiation of active proteolysis.

Attention is directed to the results of cross fermentation tests using both gas and reaction changes as criteria of the incompleteness of sugar removal and to the filtrability tests of the products of fermentation by the three organisms studied, all of which point to the advantage of using B. welchii in the preparation of sugar-free medium.

The mediums used throughout, except in exper. 7-10, consisted of beef infusion broth prepared by soaking 5 pounds of lean ground beef in 7½ liters of distilled water overnight followed by 10 minutes' boiling. The coagulated albumin was then removed by straining under pressure and paper filtration of the liquid. To the latter were then added 2% peptone (Parke, Davis and Co.) and 0.5% NaCl. This medium was adjusted to various initial reactions as indicated in the several experiments.

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[8] Ibid., 1919, 38, p. 81.

[9] Jour. Med. Research, 1911, 25, p. 117.

The mediums were put up in lots of 400 c c in 500 c c Pyrex Erlenmeyer flasks, sterilized in the autoclave, inoculated while warm and incubated at 37 C. No special means of anaerobiosis is required under these conditions to insure abundant growth of B. welchii; vigorous gas production and turbidity within a few hours gave evidence of active proliferation. Aerobic subcultures on agar slants were made at different times during the incubation period for the purpose of testing the purity of the cultures in the various flasks, and in the case of B. welchii for the purpose of detecting possible aerobic contaminants.

The determinations of the reaction at various stages of each experiment were made as follows:

The "titratable" or "total" acidity was obtained by titrating 5 c c of the medium or culture diluted with 45 c c of distilled water, to neutrality with 0.05 Normal NaOH or HCl, using phenolphthalein as indicator. The distilled water was heated to boiling prior to use to expel any carbon dioxide present.

Determinations of the H^+ ion concentration or "true" acidity were made by the colorimetric method of Clark and Lubs[10] and recorded in terms of P_H.

Exper. 1 (chart 1) was undertaken to secure frequent observations during the early growth cycles of Bact. coli and B. welchii. Readings were continued until no further variations appeared, i. e., for a total period of 8 days. B. welchii mounted to a slightly higher acid titer than Bact. coli and beyond the maxima, which were reached simultaneously in 8-24 hours; the titer of Bact. coli fell much more rapidly and reached a lower level than that of B. welchii. In neither case were there significant changes in titer after the 6th day and neither culture reached the neutral point of phenolphthalein.

The curves plotted on a basis of H^+ concentration parallel each other more closely than do the curves plotted from the titration values. The curve for both B. welchii and Bact. coli maintain a definite flat course at P_H 6.5 during 62 hours following the attainment of the maximum. It is notable that during this period, striking changes in titer were occurring, especially with Bact. coli. In this, as in subsequent experiments P_H 6.0 seems to represent the maximum H^+ ion concentration value attainable by any of the organisms studied, although in exper 7 the H^+ ion concentration was somewhat higher to start with. The possible fallacy in reading colorimetric determinations at the limit of indicator range was thoroughly appreciated and avoided throughout this work.

Exper. 2 (chart 2) duplicated exper. 1 in method with a different lot of medium. The limit of acidity as measured by both titration and P_H values was practically the same as in exper. 1, but for some reason neither value fell to so low a point as in the first instance. Peculiarly, the final titer for Bact. coli in this experiment was the same as that for B. welchii in the first, but the final titer for B. welchii was much higher than that for Bact. coli.

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[10] Jour. Bacteriol., 1917, 2, pp. 1, 109, 191.

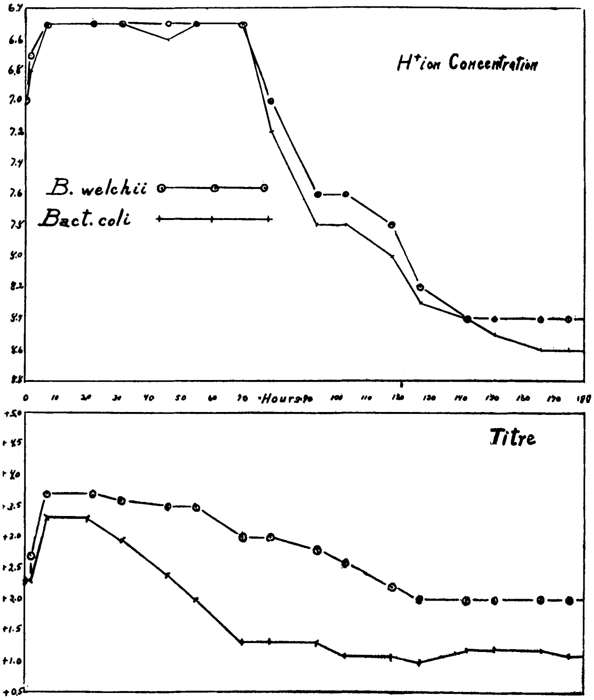

Chart 1.—Acid production of Bact. coli and B. welchii in peptone broth neutralized with NaOH, meat infusion No. 1.

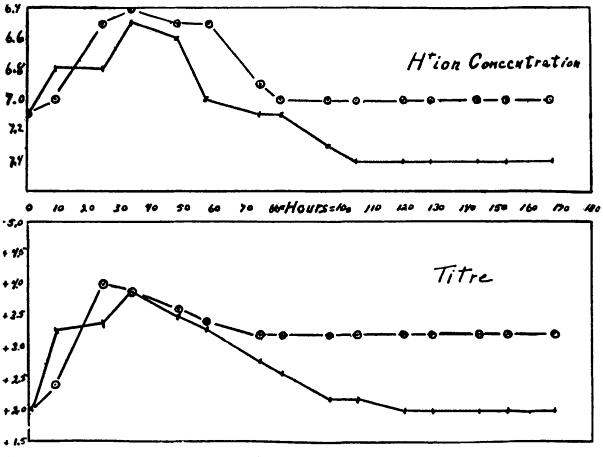

Chart 2.—Acid production of Bact. coli and B. welchii in peptone broth neutralized with NaOH, meat infusion No. 2.

The final P_H values for both organisms were also less (more acid) than in the first experiment. These differences were probably due to differnces in buffer content of the two mediums.

Exper. 3 (chart 3) was carried out with broth adjusted with 0.5% magnesium carbonate, of which the excess was removed after boiling. The essential features of the titer curves in chart 3 are similar to those of charts 1 and 2, although the peak values for both organisms are less. Again the titer for Bact. coli fell through a longer period and to a lower point than that of B. welchii. The damping effect of soluble magnesium salts is most clearly

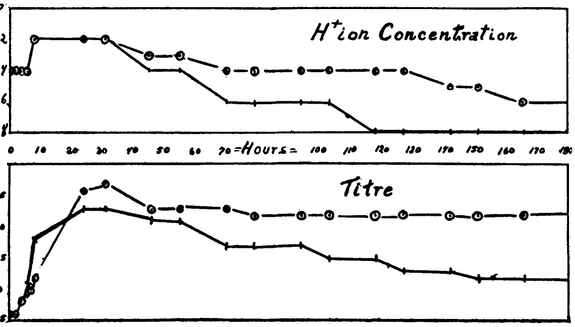

Chart 3.—Acid production of Bact. coli and B. welchii in peptone broth alkalinized with magnesium carbonate; meat infusion No. 1.

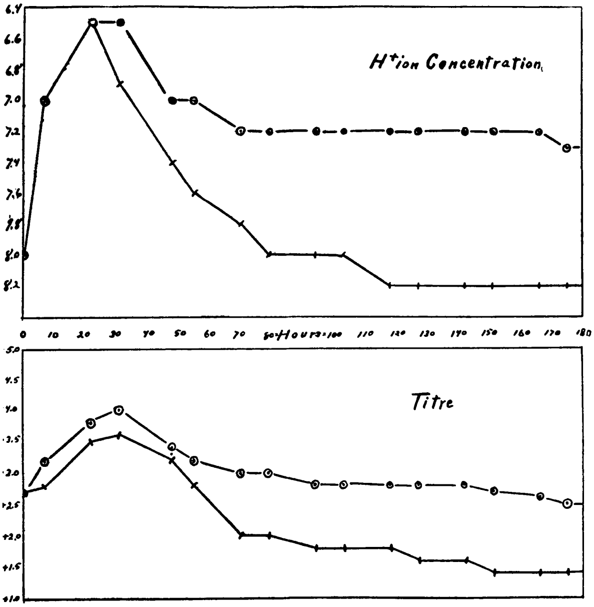

Chart 4.—Acid production of Bact. coli and B. wlechii in peptone broth alkalinized with NaOH; meat infusion No. 1.

shown in the P_H curves; those of both organisms are identical up to the 36th hour, when the acidity of both begins to fall, that of Bact. coli more rapidly.

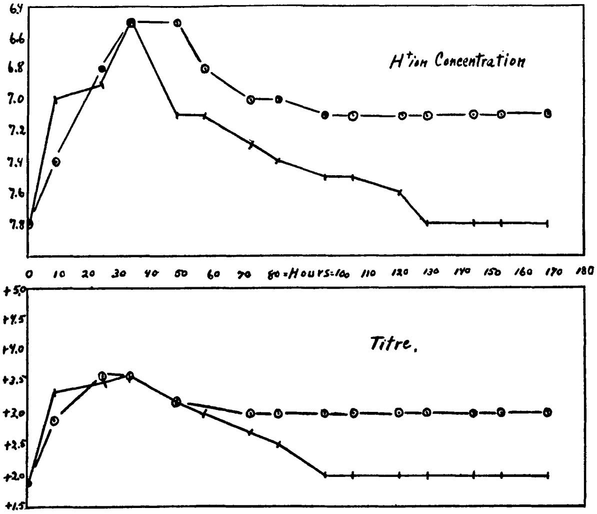

Chart 5.—Acid production of Bact. coli and B. welchii in peptone broth alkalinized with NaOH; meat infusion No. 2.

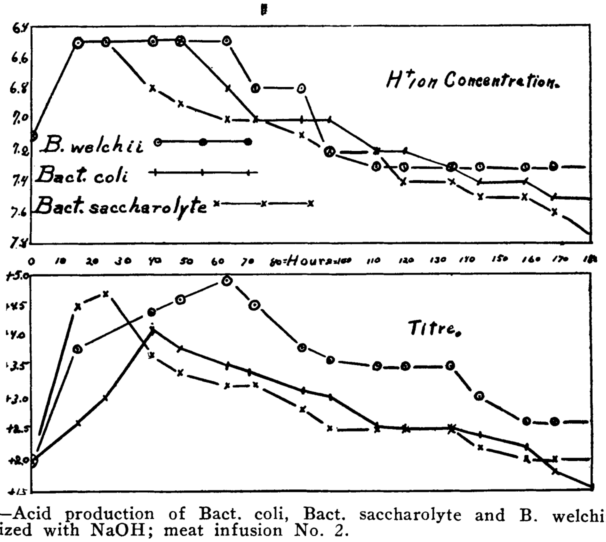

Chart 6.—Acid production of Bact. coli, Bact. saccharolyte and B. welchii in peptone broth alkalinized with NaOH; meat infusion No. 2.

Exper. 4 (chart 4) was similar to exper 1 except that the medium, which was of the same lot as in exper. 1, was adjusted to an initial P_H value of 8.0 by means of normal NaOH without making use of magnesium carbonate as in exper. 3. In spite of its distinct alkalinity as measured by the P_H value, this medium still titrated +2.7 in acidity to phenolphthalein. Inspection of the data shows, as outstanding features, that (1) the titrable acidity reached a slightly higher point in the case of B. welchii, and (2) the final P_H value and titer of B. welchii are not as low as are the corresponding values for Bact. coli.

Exper. 5 was conducted with some of the same lot of medium as exper. 2 and duplicates exper. 4 in the adjustment of initial reaction to a low P_H value with normal NaOH. Comparing charts 4 and 5, one notes the same relative differences as observed in the comparison of charts 1 and 2. Here again a possible explanation lies in the assumption of differences in buffer content of the two lots of medium.

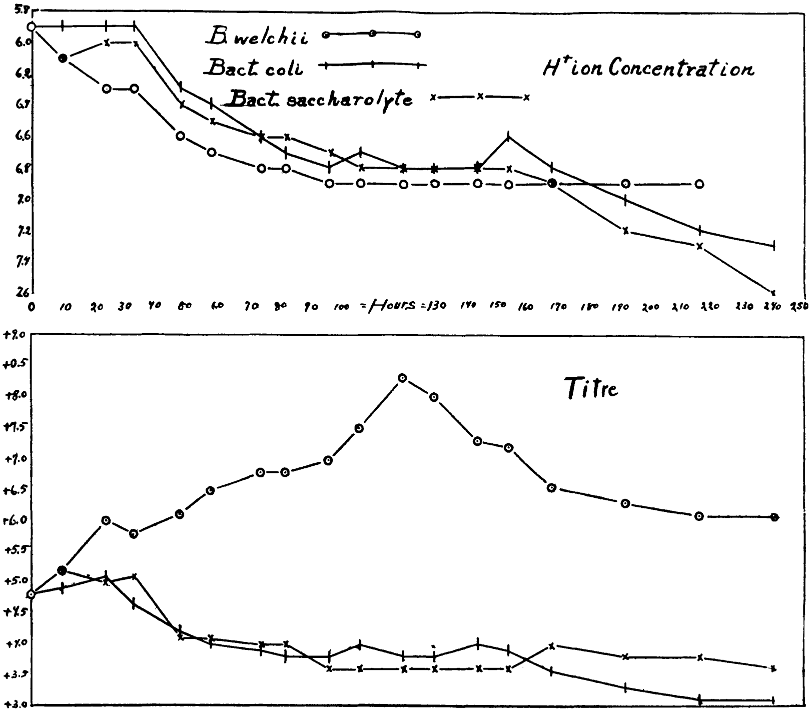

Chart 7.—Acid production of Bact. coli, Bact. saccharolyte and B. welchii in meat peptone mash.

Exper. 6 (chart 6) included Bact. saccharolyte. Judging from the titer curve, it reaches its maximum more quickly than either Bact. coli or B. welchii. The measured points extend higher than those of Bact. coli but not as high as those of B. welchii; the fall parallels that of Bact. coli; the fall of the Welch curve is slower and does not extend so low. A study of the H^+ ion concentration curve gives no additional data.

Exper. 7 (chart 7) was a study of the 3 cultures in a meat peptone mash. This medium was made by soaking the usual amount of ground lean meat, 5 pounds, in 7½ liters of distilled water, over night at room temperature, then adding 2% peptone, 0.5% NaCl and sterilizing the mash in flasks in the autoclave without straining or filtering out the coagulated albumin. No adjustment of reaction was attempted; the initial titer to phenolphthalein was therefore high, + 4.8, and the PH value was 5.9. Owing to the removal of relatively large samples (75 c c) to provide material for the acidity tests, filtration and cross refermentation tests which were carried out in this experiment, 2 flasks of 1 liter capacity well filled for each of the aerobes and 3 for B. welchii were provided in order that a change might be made from flasks in which the content of medium was becoming unduly low. No irregularities in the data secured seem to be attributable to this procedure.

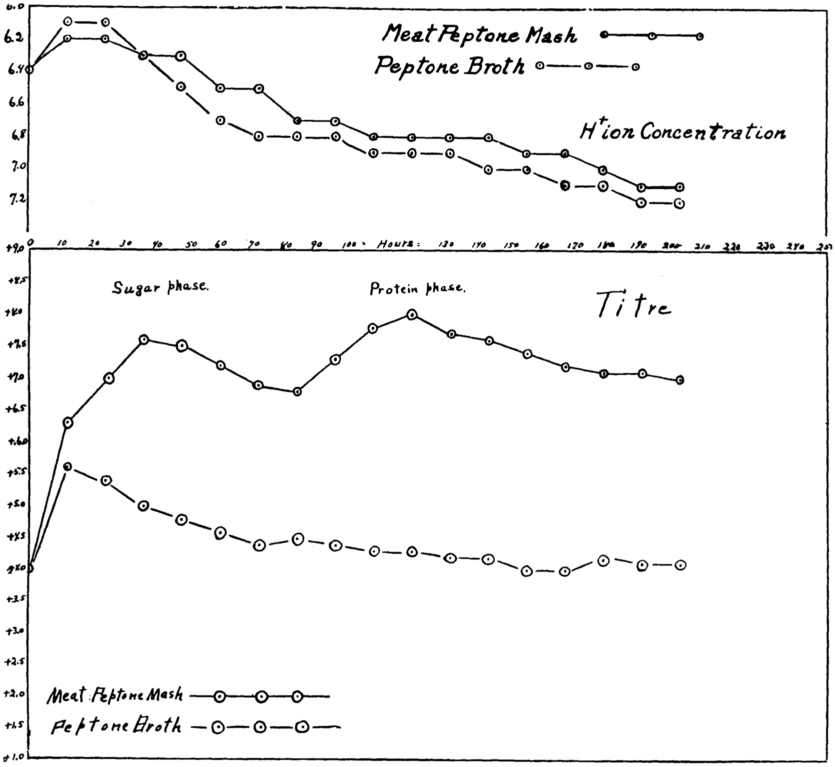

Chart 8.—Acid production of B. welchii in meat peptone mash and peptone broth.

The results differ from those in experiments in which the coagulated albumin had been removed in failing to show with any of the 3 organisms studied any initial increase in H⁺ ion concentration. This might be regarded as due to a lack of sugar in this particular lot of medium, and it is unfortunate that this experiment was not controlled with one using some of the same lot from which the coagulated albumin had been removed. We were inclined to believe the high buffer content of the medium served to mask the production of acid, or that perhaps the continuous fall in the H⁺ ion concentration represented a predominance of the protein metabolism over a possible sugar metabolism due to the relative preponderance of protein in this medium. It was noted that after Bact. coli and Bact. saccharolyte had apparently reached a level between P_H 6.6 and 6.8 at the 80th hour of incubation these 2 cultures began a new depression of the H^+ ion concentration at the 170th hour. This did not occur with B. welchii. The titration curves are most striking in this experiment. After a brief initial rise in titratable acidity, the curves of all 3 organisms fell as usual. But that for B. welchii shortly began to ascend to a peak which it reached after the first 120 hours of incubation, when it fell distinctly.

We thought at first that the increase in titratable acidity might be attributed to an actual production of acids which failed to increase the H^+ ion concentration due to their immediate neutralization by the high protein buffer content of the medium. This experiment presented the apparent enigma of a culture increasing its titratable acidity while actually becoming more alkaline as measured in terms of P_H. But the paradox disappeared when it was remembered that phenolphthalein was used as the indicator in the titration and that the point of reference, i. e., the changing P_H of phenolphthalein, is about 9.

We were confronted in this experiment with a choice of criteria for our judgment as to when such a medium is sugar free. It seemed that the H^+ ion concentration data yielded nothing of value here. And if we relied on the turn in the titratable acidity curves to indicate the sugar-free point, which organisms was to be regarded as yielding reliable data? There was no question that the decision rested on the true interpretation of the remarkable result with B. welchii. If the peak of the Welch titration curve were to be accepted as the sugar-free point, a much longer time would have to be allowed than is customary for freeing medium from sugar when the coagulable proteins are not removed.

We undertook to settle these points by comparing the acid production of B. welchii in meat peptone mash with that in some of the same medium from which the coagulated meat had been removed by filtration. This experiment was performed twice with essentially similar results, which are shown for one of the trials in chart 8.

The usual initial rise in H^+ ion concentration and titer was observed irrespective of the presence of excess of meat. This confirmed our belief that the medium used in exper. 7 was deficient in sugar. The damping effect of the meat mash medium is a feature in the H^+ ion concentration curves; a distinctly wider range of P_H values was recorded for the peptone broth medium.

The initial peak in the titration curve in the meat mash medium, followed by a secondary and higher peak as observed in exper. 7 was accentuated in exper. 8. The lack of any secondary rise in titer in the more lightly buffered peptone broth suggested that the secondary rise in titer in the meat mash medium was due to the admittedly weak proteolytic action of B. welchii on undigested protein. We have interpreted the first peak as the phase of sugar metabolism, the second, the phase of protein metabolism.

The experiments support our belief that a change in the direction of greater H^+ ion concentration (lower P_H value) constitutes the best criterion of fermentation; titration indicates not only acidity but may indicate increased buffer capacity brought about by proteolysis and is therefore an unsafe guide in determining the point at which a given medium can be said to be free from sugar.

REFERMENTATION TESTS

Most bacteriologists regard the sugar effectively removed from a medium when it fails to produce gas on refermentation with Bact. coli. It occurred to us that some information might be secured by cross fermentation tests between the three cultures here represented.

The samples removed from the flasks in exper 7 were utilized for the purpose, being first filtered through paper, and then tubed in the constricted fermentation tube with marble seal designed by one of us and sterilized in the autoclave. When the last of the samples in exper 7 were so collected all were inoculated and incubated at 37 C. for 4 days. Each tube was examined for contamination by subcultures of plain agar.

It was striking that none of the refermentation tests of the Welch broth gave gas. It must be admitted that the Welch bacillus itself failed to grow, unless we can regard the slight changes in reaction as indicative of growth. We note that these changes were in the direction of greater acidity above the heavy line in the table, which marks the peak of the titer in the Welch broth and in the direction of greater alkalinity below that line.

In the case of Bact. coli and Bact. saccharolyte all of the tests showed increased alkalinity—even in those samples removed before the peak of the Welch titer had been reached. Thus there is no evidence of a failure of B. welchii to remove all the fermentable sugar from the medium.

Contrasting the behavior of Bact. coli broth with B. welchii broth we note that growth occurred in all samples tested. In all cases but one, that of the 48 hour broth, B. welchii produced an increase in acidity and in all but the 24 hour and 48 hour samples, rather abundant gas. The data indicate the incomplete removal of sugar by Bact. coli. Yet the colon bacterium failed to produce gas in any of the samples and all became more alkaline, as might be expected from the fact that the peak of the acid titer was reached in the colon broth at about 24 hours. The same was true of the refermentation of the colon broth by Bact. saccharolyte except that in one instance a small amount of gas was produced.

The observations made for the colon broth apply in the main also to the saccharolyte broth except that no growth of B. welchii occurred in samples less than 48 hours old. Both acid and gas were produced by B. welchii, showing the incompleteness of sugar removal by Bact. saccharolyte. Yet this broth would be considered satisfactory from the standpoint of refermentation by Bact. coli and Bact. saccharolyte, neither of which produced acid or gas.

FILTRATION EXPERIMENTS

If a clear "sugar-free" medium is desired after the use of Bact. coli or Bact. saccharolyte in its preparation, filtration through paper is not sufficient to give a satisfactory product; adsorbents, such as talc and diatomaceous earth, may, however, be used as clearing agents. While we have frequently had occasion to utilize adsorbents in clearing mediums, their investigation is not a part of the present communication. We were interested in comparing the filtering properties of cultures of B. welchii with those of Bact. coli and Bact. saccharolyte with respect to rapidity of filtration and clarity of product.

Exper. 9: The autoclaved cultures from exper. 3 were used. One hundred c c portions were taken from each flask after thorough agitation of the contents. The two portions of killed culture were then poured simultaneously into similar funnels containing the same size and type of filter paper. The filtrates were collected in graduated cones. The colon cultures passed through the paper more rapidly than did the culture of B. welchii, but the filtrate obtained from the latter was far superior to the colon filtrate as regards clarity.

Exper. 10: In this experiment the cultures were filtered without previous agitation. This time the Welch culture passed through the paper more rapidly than the colon culture. The quality of the filtrate favored the Welch product in clarity.

Exper. 11: Twenty-four hour broth cultures (400 c c each) of B. coli and B. welchii were autoclaved for 20 minutes at 15 pounds pressure. Immediately after the pressure was down and without agitation, 100 c c portions of each culture were removed and filtered simultaneously through one thickness of medium grade filter paper. The colon culture passed through the paper twice as rapidly as did the Welch culture, but the filtrate obtained was not nearly as clear as that obtained from B. welchii. Both filtrates were then refiltered; this time the Welch filtrate passed through the paper faster than the coli filtrate. The second filtrate of B. welchii was as clear as ordinary beef broth whereas the Bact. coli filtrate was still quite murky and required the use of a clearing agent to bring it to a parity with the Welch product. This experiment was repeated with identical results.

At this time a more serious consideration of the filtrability problem was conceived in order to detect any possible relation of filtrability to the stage of fermentation in which incubation was stopped. Thus the samples removed in exper. 7 were utilized as follows:

Exper. 12: Constant volumes (75 c c) of the unheated samples were filtered through as uniform as possible pieces of filter paper.

It is seen that the initial filtrability of B. welchii broth was low during the early stages of growth in meat peptone mash but increased as the culture became older, and especially after it passed the peak of the titration curve. The mucoid tenacity of this culture when young may account for the slowness of filtration at that stage of its growth.

The filtrability of Bact. coli broth was high and became better as the culture aged; that of Bact. saccharolyte was at a maximum and approximately equivalent to that of B. coli during the first 24 hours, after which it became progressively poorer.

The clarity of the products of filtration of Bact. coli and Bact. saccharolyte was at all times inferior to that of B. welchii.

We recognize the limitation of these observations to meat peptone mash; mediums with a lower protein content and particularly a lower coagulable albumin content might readily give different results.

SUMMARY

In this paper we propose the use of B. welchii in the preparation of "sugar-free" broth. This suggestion grows out of the well-known vigorous fermentative ability of this organism. It is suggested that the fermentation be conducted with culture medium to which all of the protein substances to be used in it have been added. No special means of providing anaerobiosis for the growth of B. welchii in such mediums are necessary other than inoculation before the medium is cool after sterilization, since the depth in quantities such as are ordinarily used provides an adequate exclusion of air. After growth starts the evolution of gas produced by fermentation automatically removes oxygen.

The fermentation should be followed by means of acidity determinations. Both on theoretical and practical grounds an increase in H^+ concentration provides the best criterion of sugar splitting. When the protein buffer content of the medium is high, titratable acidity is an unsafe guide in determining when a given medium can be said to be free from fermentable sugar. In the present state of our knowledge of the use of both methods of acidity determination from which the acid curves may be plotted would seem superior to the present rule of thumb methods.

We have not been able to use chemical sugar tests of sufficient delicacy to detect traces of sugar in broth which can be demonstrated by cross fermentation tests. The results of cross fermentation tests, on the contrary, support the interpretation of the acidity tests proposed. Cross fermentation tests show that B. welchii is still able to produce acid and gas from mediums presumably exhausted by Bact. coli and Bact. saccharolyte. These organisms are not able to produce either acid or gas in mediums fermented out with B. welchii. This fact indicates the greater completeness of fermentation by B. welchii and may actually point to the existence in broth of carbohydrates not fermented by the aerobes studied, possibly more complex sugars than the monosaccharides.

The time required for elimination of sugar by B. welchii, Bact. coli, and Bact. saccharolyte is approximately equal. But saccharolyte generally produces its maximum acidity in the shortest time—always less than 30 hours in the mediums studied. There is no choice among these organisms from this standpoint.

While the "sugar-free" broth produced by B. welchii filters through paper more slowly than that of Bact. coli and Bact. saccharolyte in the early stages of fermentation, its filtrability improves as the culture ages, and the product is at all times much clearer than that of Bact. coli and Bact. saccharolyte so that the use of adsorbents for clarification is unnecessary.